Award Number: W81XWH-13-1-0040

TITLE: Role of CTGF in White Matter Development in Tuberous Sclerosis

PRINCIPAL INVESTIGATOR: Mustafa Sahin

CONTRACTING ORGANIZATION: The Children's Hospital Corporation
Boston, MA 02115

REPORT DATE: February 2015

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
FEB 2015		**01-02-2014 to 31-01-2015**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Role of CTGF in White Matter Development in Tuberous Sclerosis	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **The Children's Hospital Corporation,,Boston,,MA,02115**	8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Our results show that neurons lacking Tsc1 results in a block in oligodendrocyte maturation and thus hypomyelination in the mouse brain. We show that Tsc-deficient neurons secrete excessive amounts of connective tissue growth factor (CTGF), which blocks the maturation of oligodendrocytes. We show here that CTGF is both necessary and sufficient to block the oligodendrocyte maturation. To determine whether CTGF is necessary in vivo, we generated mice lacking both Tsc1 and CTGF in neurons (Tsc1cc;CTGFf/f;SynCre+) mice. In order to investigate the effect of CTGF on oligodendrocyte maturation, we propose to treat the oligodendrocytes with different domains of CTGF (Module I to IV). Moreover, in order to investigate the upstream regulation of CTGF expression in Tsc-deficient neurons, we tested Serum Response Factor (SRF) pathway, which was previously shown to affect CTGF expression. Our preliminary results show that SRF is downregulated in Tsc1 mutant brains and this can be rescued by rapamycin treatment suggesting a crosstalk between SRF and mTOR pathways.

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**	**Same as Report (SAR)**	**11**	

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
february 2015	Annual	1 Feb 2014 - 31 Jan 2015

4. TITLE AND SUBTITLE
Role of CTGF in White Matter Development in Tuberous Sclerosis

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-13-1-0040

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Mustafa Sahin

email: mustafa.sahin@childrens.harvard.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

The Children's Hospital Corporation
Boston, MA 02115

8. PERFORMING ORGANIZATION REPORT

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Our results show that neurons lacking Tsc1 results in a block in oligodendrocyte maturation and thus hypomyelination in the mouse brain. We show that Tsc-deficient neurons secrete excessive amounts of connective tissue growth factor (CTGF), which blocks the maturation of oligodendrocytes. We show here that CTGF is both necessary and sufficient to block the oligodendrocyte maturation. To determine whether CTGF is necessary *in vivo*, we generated mice lacking both Tsc1 and CTGF in neurons ($Tsc1^{cc}$;$CTGF^{f/f}$;$SynCre^{+}$) mice. In order to investigate the effect of CTGF on oligodendrocyte maturation, we propose to treat the oligodendrocytes with different domains of CTGF (Module I to IV). Moreover, in order to investigate the upstream regulation of CTGF expression in Tsc-deficient neurons, we tested Serum Response Factor (SRF) pathway, which was previously shown to affect CTGF expression. Our preliminary results show that SRF is downregulated in Tsc1 mutant brains and this can be rescued by rapamycin treatment suggesting a crosstalk between SRF and mTOR pathways.

15. SUBJECT TERMS
Tuberous Sclerosis Complex, myelination, CTGF

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	11	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

	Page
1. Introduction……	**4**
2. Keywords……	**4**
3. Accomplishments……..……...	**4**
4. Impact……...……	**5**
5. Changes/Problems...…..……	**6**
6. Products…….…….…….……	**6**
7. Participants & Other Collaborating Organizations……	**6**
8. Special Reporting Requirements……	**6**
9. Appendices……	**7**
10. References……	**11**

INTRODUCTION

Tuberous sclerosis complex (TSC) is an autosomal dominant multisystem disorder caused by loss of either TSC1 or TSC2 function (Tsai and Sahin, 2011). TSC affects 1/6,000 individuals worldwide and affects multiple organs including the brain, skin, eyes, kidneys, heart, and lungs (Crino et al., 2006). TSC patients present with epilepsy (~90%), intellectual disability and autism (~50%), and other disorders including sleep disruption, attention-deficit hyperactivity disorder, and anxiety(Han and Sahin, 2011). The neuropathological findings in TSC are cortical tubers, subependymal nodules and subependymal giant cell astrocytomas (SEGAs) (DiMario, 2004). Another important yet not well-studied feature of TSC pathology in brain is hypomyelination (Ridler et al., 2001). Most recently using diffusion tensor imaging we observed abnormal white matter microstructure in patients with TSC that have autism compared to TSC patients without autism(Lewis et al., 2013; Peters et al., 2013). To uncover the underlying molecular mechanisms of hypomyelination in TSC, we investigated the role of neuronal factors affecting oligodendrocyte development in our *Tsc1^{cc}SynICre^{+}* mouse model, which lacks Tsc1 expression only in neurons. Here we show that, neurons lacking Tsc1 secrete excessive amounts of connective tissue growth factor (CTGF), which in turn blocks the maturation of oligodendrocytes, and thus myelination both *in vitro* and *in vivo*.

KEYWORDS

Tuberous Sclerosis Complex, myelination, CTGF

ACCOMPLISHMENTS:

We will review the accomplishments to date in each aim, as taken from the SOW (in **bold**). The figures are in the appendices.

Aim 1A: To determine the role of CTGF in hypomyelination in the mouse model.

I. We will generate two independent *CTGF*-shRNAs and confirm their efficacy in vitro.

We had previously shown that supernatant from HEK293T cells that express full length CTGF or commercially available recombinant CTGF reduce the number of MBP+ oligodendrocytes in cultures (Figure 1a-d). In addition, we had showed that adding neutralizing anti-CTGF antibody to conditioned medium from TSC-deficient neurons was able to ameliorate the effect of this conditioned medium on oligodendrocyte maturation and MBP expression (Figure 1e-f). We had proposed knocking down CTGF in TSC-deficient neurons as one way to verifying the role of CTGF in vitro. We did not proceed with these in vitro experiments since the in vivo experiments detailed below gave extremely compelling results allowing us to focus on the role of CTGF in the mouse model.

II. We will generate AAV2-CTGF-shRNA to reduce the expression of CTGF in vivo. AAV2-expressing scrambled shRNA as a control to the contralateral hemisphere. Alternatively, we will generate mice missing both Tsc1 and Ctgf genes under the Syn-Cre promoter (see grant proposal for details). Then we will follow look at MBP staining in the double knockout mice compared to single Tsc1 knockout mice.

We crossed *Tsc1;SynICre^{+}* mice with *CTGF$^{f/f}$* mice (Kapoor et al., 2008) to generate the following groups of mice: wild type for both genes (*Tsc1^{ww};SynICre^{+};CTGF$^{+/+}$*), mutant for *Tsc1* and wild type for *CTGF* (*Tsc1^{cc};SynICre^{+};CTGF$^{+/+}$*) and mutant for both genes (*Tsc1^{cc};SynICre^{+};CTGF$^{f/f}$*). To our knowledge, this is the first mouse model lacking CTGF only in neurons. We stained the brain sections of control, mutant and double mutant with the MBP antibody to visualize myelination and found that loss of CTGF partially rescued the hypomyelination phenotype as assessed by the increased MBP

signal (Figure 1g-h). Interestingly, the number of mature oligodendrocytes, which were counted by CC1 staining, did not change between the mutant and double mutant (Figure 2a-c). We used CC1 expression to detect the mature oligodendrocytes by immunohistochemistry. This suggests that CTGF blocks the differentiation of oligodendrocytes to myelinating oligodendrocytes; however, another yet unknown factor affects the development of oligodendrocytes in mice lacking Tsc1 in neurons. Moreover, the loss of CTGF in neurons, which are wild type for *Tsc1*, also enhanced myelination (Figure 2d-f). Together these data suggest that the neuronal CTGF is necessary for blocking myelination *in vivo*.

These results beg the question how TSC-deficiency regulates CTGF expression. We focused on the role of SRF. Previous reports show that the SRF functions as the repressor of *Ctgf* transcription (Stritt et al., 2009). Importantly, a previous study showed that neurons lacking SRF have higher expression of CTGF and thus blocks the maturation of oligodendrocytes (Stritt et al., 2009). We therefore analyzed the levels of SRF both in our mutant mice and in primary cortical neurons lacking *Tsc2*. Both SRF protein and transcript levels were decreased in *Tsc2* KD neurons compared to the control neurons (Figure 3a-c). In addition, we checked the transcript levels of other targets of SRF, *Egr1* and *Cyr61*. SRF functions as a transcription activator of *Egr1* (Kim et al., 2008), whereas it suppresses the transcription of *Cyr61* (Stritt et al., 2009), which is in the same CCN family of proteins as CTGF (Jun and Lau, 2011). In Tsc2 KD cortical neurons, both the transcript levels of SRF and *Egr1* are decreased, whereas *Cyr61* is increased as *Ctgf* (Figure 3c). Moreover, when stained with SRF antibody, compared to the control mice, the mutant mice brain sections showed decreased intensity, suggesting SRF expression is diminished both *in vivo* and *in vitro.* Together, our data suggest that the upregulation of CTGF in *Tsc*-deficient neurons may be due to the downregulation of it repressor, SRF.

<u>Aim 1B:</u> To test whether CTGF expression is altered in human TSC brain.

We have initiated staining of CTGF in tuber specimens from TSC patients taken at the time of epilepsy surgery. We have so far stained paraffin sections of tubers from TSC patients and detected that CTGF is expressed in the tuber. We found that all phosphor-S6 positive cells are also CTGF-positive (Figure 4).

<u>Aim 2:</u> To examine the mechanisms by which CTGF regulates oligodendrocyte differentiation.

Our preliminary data from our first-year report suggests that the Mod-IV, which is one of the domains of CTGF is responsible for inhibiting oligodendrocyte maturation. To address this question systematically, we have been generating CTGF expression constructs, which express different domains of CTGF (Mod-I, Mod-II, Mod-III, Mod-IV and their combinations) to test the mutual and/or complementary effects of these different domains on oligodendrocyte maturation. So far we have generated the following FLAG-tagged constructs: Full-length, Module I, Module I+II. The cloning for the rest of the constructs is in progress. We had showed that the CM collected from HEK293T cells expressing the full-length FLAG-CTGF affects the maturation of oligodendrocytes. We will now test the individual modules for their effect on oligodendrocyte maturation assay.

IMPACT

In this study, we report that the double mutant, which lacks both Tsc1 and CTGF in neurons rescue myelination. Moreover, we continue to work on the upstream regulators of CTGF expression and we focused on SRF pathway. In addition to our preliminary findings on SRF pathway in our previous report, here we show that SRF levels are decreased in vivo in mutant mice, and this can be rescued by rapamycin treatment. The crosstalk between SRF and mTOR pathways will not only provide a better understanding of regulation of CTGF expression but also help us to understand the basic control mechanisms of cell growth and survival. Better understanding of the role of CTGF in

myelination may provide insights and new therapeutic options for TSC and other diseases affecting the white matter.

CHANGES/PROBLEMS:
None

PRODUCTS:
We submitted the manuscript describing our findings for publication.

We have also published two review papers related to this topic and acknowledged the funding from the DOD:

Autism and the synapse: emerging mechanisms and mechanism-based therapies.
Ebrahimi-Fakhari D, **Sahin M.**
Curr Opin Neurol. 2015 Feb 19. [Epub ahead of print]
PMID: 25695134

Davis PE, Peters JM, Krueger D and **Sahin M.** Tuberous Sclerosis: A new frontier in targeted treatment of autism. Neurotherapeutics (in press).

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS
None

SPECIAL REPORTING REQUIREMENTS
None

APPENDICES:
Figures 1-4

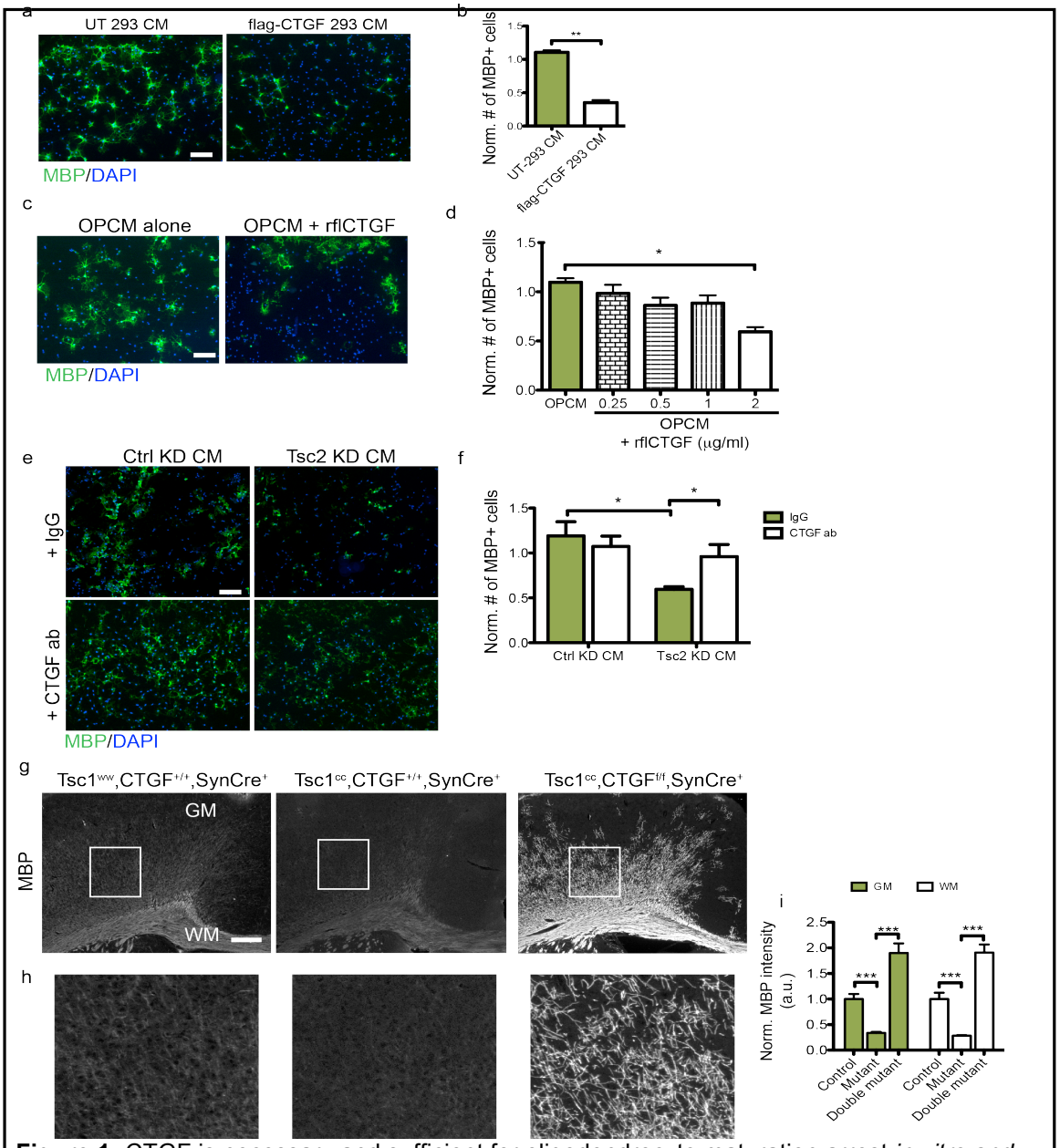

Figure 1: CTGF is necessary and sufficient for oligodendrocyte maturation arrest *in vitro and in vivo*. a) OPCs, which are treated by either condition medium from untransfected HEK293T cells (UT 293T CM) or condition medium from HEK293T cells expressing flag-CTGF (flag-CTGF CM) are stained for MBP (green) and Hoechst (blue). b) Quantifications of the cell numbers normalized to Hoechst+ cells (N=3). c) OPCs are grown in OPC medium (OPCM) or OPCM containing 2 μg/ml are stained for MBP (green) and Hoechst (blue). d) Quantifications of the MBP+ cells treated with increasing doses recombinant full length CTGF (rfl-CTGF) normalized to total cell number (Hoechst+ cells) (n=4) e) OPCs, which are treated by either control KD CM or Tsc2 KD CM are treated with either IgG or CTGF antibody (ab) and stained for MBP (green) and Hoechst (blue). f) Quantifications of the cell numbers in (e) normalized to Hoechst+ cells (total cell number) and MBP+ cells treated with CM from non-infected neurons (n=5). g) MBP staining of brain sections from control (n=5), mutant (n=6) and double mutant (n=4) mice and h) Enlarged images corresponding to the area depicted with white square in (g). i) MBP signal quantifications of corresponding mice for GM WM in arbitrary units (a.u.).

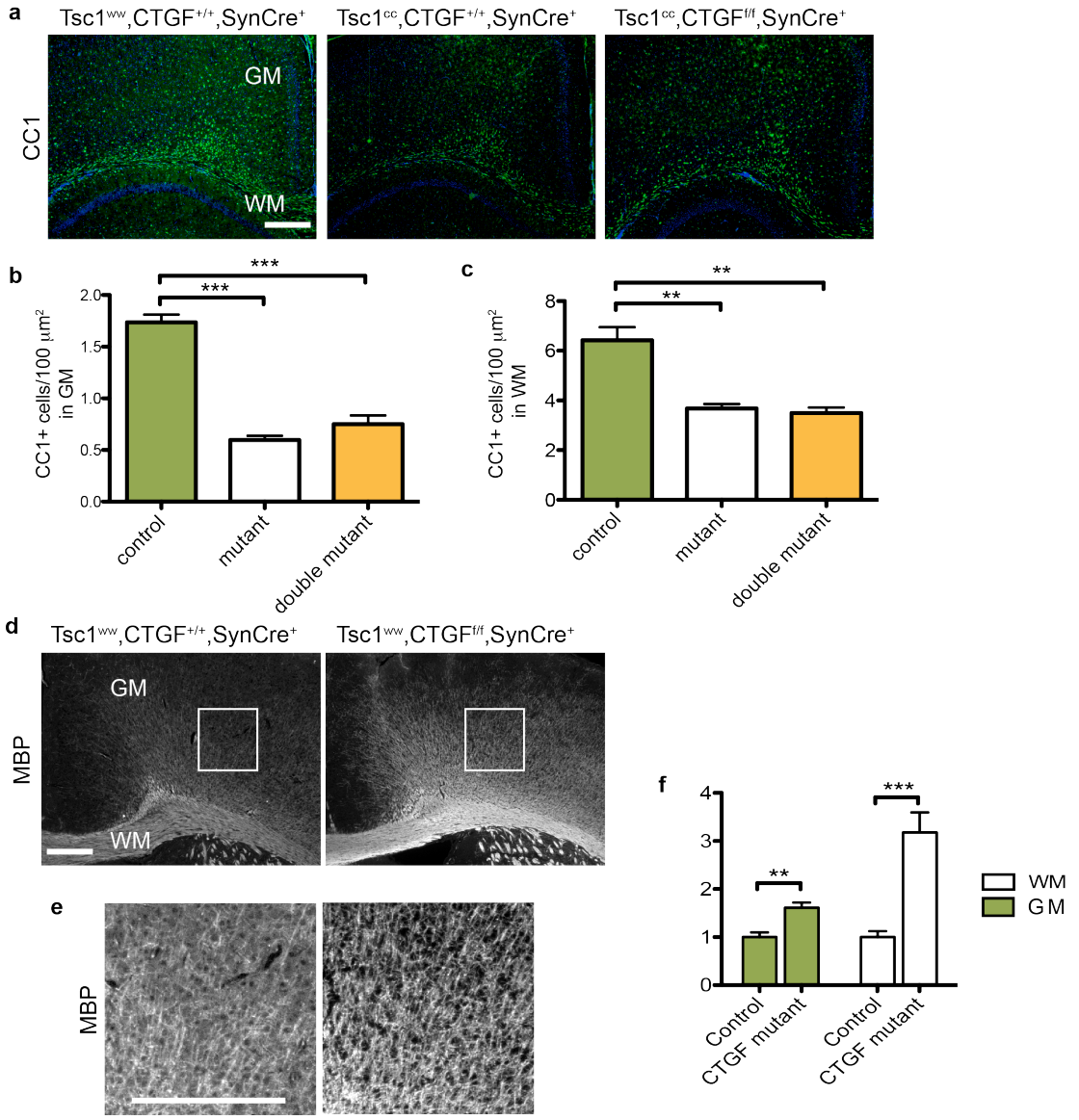

Figure 2. The number of CC1 positive mature oligodendrocyte number decreases in the Tsc1 mutant mice, the double mutant, which lacks both Tsc1 and CTGF does not rescue the number of CC1 positive cells (a-c). Mice, which are wild type for Tsc1 but lacking CTGF in neurons, represent higher myelination as MBP intensity increases in the CTGF mutant compared to the wild type control (d-f).

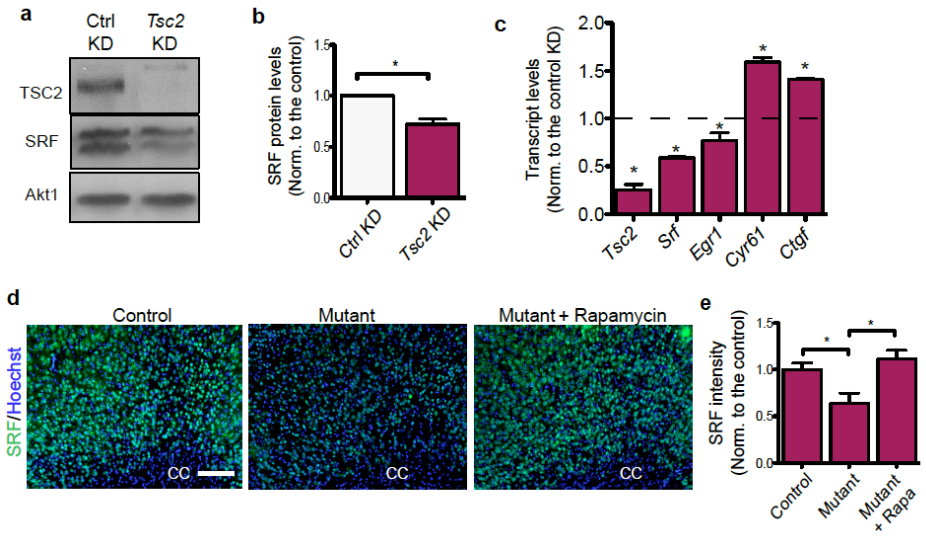

Figure 3. SRF is downregulated in *Tsc*-deficient neurons. a) Immunoblotting of control and *Tsc2* KD cortical neurons showing Tsc2, SRF and Akt1 (loading control). b) Quantification of the SRF protein levels normalized to Akt1 (n=3). c) qRT-PCR of *Tsc2, Srf, Egr1, Cyr61* and *Ctgf* in *Tsc2* KD primary cortical neurons (n=3), showing the transcript levels. The dashed line represents the transcript levels in the control KD, which is set to 1. d) SRF staining of brain sections from control (n=3), *Tsc1* mutant (n=3) and rapamycin treated mutant (n=3) mice, scale bar corresponds to 200μm and corpus callosum (CC) is depicted. e) Quantifications of SRF intensity of the corresponding mice in arbitrary units (a.u.). (*p<0.05).

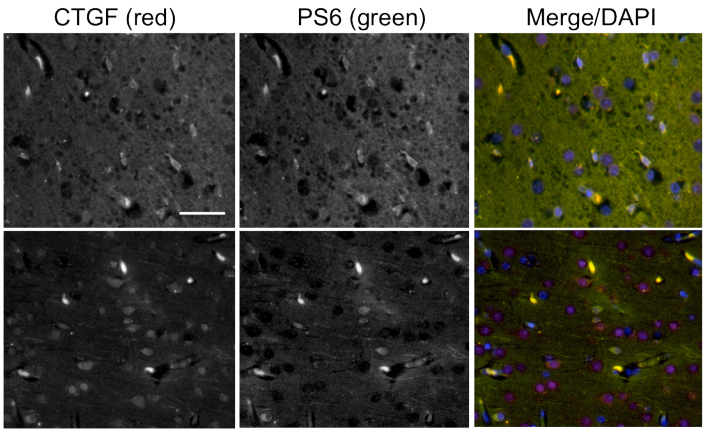

Figure 4. CTGF is expressed in tuber tissue. Two paraffin sections of a tuber from a TSC patient are stained with CTGF (red) and PS6 (green) antibodies. Nuclei are stained with Hoechst (blue). All PS6 positive cells are positive for CTGF. Not all CTGF positive cells are PS6 positive. Scale bar represents 100μm.

REFERENCES

Crino, P.B., Nathanson, K.L., and Henske, E.P. (2006). The tuberous sclerosis complex. N Engl J Med 355,1345-1356.

DiMario, F.J., Jr. (2004). Brain abnormalities in tuberous sclerosis complex. J Child Neurol 19,650-657.

Han, J.M., and Sahin, M. (2011). TSC1/TSC2 signaling in the CNS. FEBS Lett 585,973-980.

Heuer, H., Christ, S., Friedrichsen, S., Brauer, D., Winckler, M., Bauer, K., and Raivich, G. (2003). Connective tissue growth factor: a novel marker of layer VII neurons in the rat cerebral cortex. Neuroscience 119,43-52.

Jun, J.I., and Lau, L.F. (2011). Taking aim at the extracellular matrix: CCN proteins as emerging therapeutic targets. Nat Rev Drug Discov 10,945-963.

Kapoor, M., Liu, S., Huh, K., Parapuram, S., Kennedy, L., and Leask, A. (2008). Connective tissue growth factor promoter activity in normal and wounded skin. Fibrogenesis Tissue Repair 1,3.

Kim, M.J., Kang, J.H., Chang, S.Y., Jang, H.J., Ryu, G.R., Ko, S.H., Jeong, I.K., Kim, M.S., and Jo, Y.H. (2008). Exendin-4 induction of Egr-1 expression in INS-1 beta-cells: interaction of SRF, not YY1, with SRE site of rat Egr-1 promoter. J Cell Biochem 104,2261-2271.

Lewis, W.W., Sahin, M., Scherrer, B., Peters, J.M., Suarez, R.O., Vogel-Farley, V.K., Jeste, S.S., Gregas, M.C., Prabhu, S.P., Nelson, C.A., 3rd, and Warfield, S.K. (2013). Impaired language pathways in tuberous sclerosis complex patients with autism spectrum disorders. Cereb Cortex 23,1526-1532.

Peters, J.M., Taquet, M., Prohl, A.K., Scherrer, B., van Eeghen, A.M., Prabhu, S.P., Sahin, M., and Warfield, S.K. (2013). Diffusion tensor imaging and related techniques in tuberous sclerosis complex: review and future directions. Future Neurol 8,583-597.

Ridler, K., Bullmore, E.T., De Vries, P.J., Suckling, J., Barker, G.J., Meara, S.J., Williams, S.C., and Bolton, P.F. (2001). Widespread anatomical abnormalities of grey and white matter structure in tuberous sclerosis. Psychol Med 31,1437-1446.

Stritt, C., Stern, S., Harting, K., Manke, T., Sinske, D., Schwarz, H., Vingron, M., Nordheim, A., and Knoll, B. (2009). Paracrine control of oligodendrocyte differentiation by SRF-directed neuronal gene expression. Nat Neurosci 12,418-427.

Tsai, P., and Sahin, M. (2011). Mechanisms of neurocognitive dysfunction and therapeutic considerations in tuberous sclerosis complex. Curr Opin Neurol 24,106-113.